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Identification of the High-Virulence Clone of Group B Streptococci in Mexican Isolates by Growth Characteristics at 40°C

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Abstract. Group B streptococci (GBS) colonizing the vagina and rectum of pregnant women cause invasive disease of the offspring in a small number of cases. The immune status of the host and differences in virulence among strains appear to be the main determinants for neonatal infection. A high-virulence clone (HVC) was proposed to cause much of the morbidity and mortality when a collection of GBS isolates was examined by multilocus enzyme electrophoresis. HVC isolates could be further distinguished by their inability to grow at 40°C. This characteristic was used in the present study to examine a collection of 57 GBS isolates from Mexico City for the HVC. Three serotype III invasive strains were classified in the HVC. The other eleven invasive strains and all carrier isolates had growth curves unaffected at 40°C. These results demonstrate the presence of the HVC in Mexico. Such a low prevalence could explain in part the low rate of GBS invasive neonatal disease in Mexico.

GBS (*Streptococcus agalactiae*) are the most common cause of neonatal invasive disease in the United States and other developed countries [18, 20, 28], where serotype III accounts for more than two-thirds of all GBS-related neonatal disease cases [1, 26]. Because of that, serotyping was proposed as a mean of predicting the risk of invasive disease; however, serotype III is also frequent in asymptomatic carriage, and serotypes other than type III are frequently isolated in early-onset disease [1]. Therefore, though serotyping has been the traditional method used to classify GBS isolates, the discriminatory power of this scheme is undesirably low [4]. In addition, as many as 10–15% of GBS isolates have been nontypable in some studies [1, 4, 21], making comparisons with other serotype isolates impossible. A number of molecular methods, such as multi-locus enzyme electrophoresis, restriction endonuclease analysis of chromosomal DNA, and DNA restriction fragment length polymorphism analysis have been used in epidemiologic investigation for subtyping GBS isolates and correlating particular genotypes with disease. Nevertheless, there is as yet no system that can adequately subtype GBS and predict disease [2, 4, 8, 9, 19].

On the other hand, although strains of GBS colonizing the genital tract can invade amniotic fluid before or during labor, not all strains are able to cause invasive disease in the newborn. Therefore, vaginal colonization should not be systematically considered a risk for neonatal infection, and the use of antibiotics for vaginal carrier state is probably unnecessary in many cases [1, 9, 19, 28]. At the present time, it is not known why some individuals develop disease, whereas the majority remain unaffected. Differences in the immune status of the host as well as variation in virulence among GBS strains may determine the outcome of colonization and infection [1, 15, 19, 20].

Musser et al. [15] in a population genetic study of 128 isolates of GBS by multi-locus enzyme electrophoresis found that strains synthesizing type III polysaccharide belonged to two distantly related evolutionary lineages, which were apparently different in their pathogenic potential. A single clonal type (phylogenetic division I) with an unusually high degree of virulence was responsible for most of the morbidity and mortality caused by type III GBS isolates, and was proposed to be an HVC of GBS. Similar results were found by Helmig et al. [9] in

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Denmark and Quentin et al. [19] in France. Their results confirmed the clonal structure of the population of GBS, the differences in virulence among clones, the phylogenetic dichotomy of type III isolates, and the existence of a limited number of clones causing invasive neonatal disease. However, a recent study evaluating 23 genetic and phenotypic characteristics in 91 human GBS isolates failed to find differences in pathogenic potential among clonal types [8].

In efforts to develop specific diagnostic procedures to identify the HVC of GBS, several unique characteristics have been found, including its inability to grow at 40°C in a chemically defined medium [12]. This characteristic has been used as a highly specific and sensitive marker to identify this clone [11, 24].

Even though GBS are considered an uncommon cause of perinatal infections in Mexico [3, 22], recent studies found vaginal colonization rates of 10% in pregnant women [21] and a neonatal infection rate of 1/1500 live births with a high case-fatality rate [22]. In addition, Palacios et al. [17] recently reported that type III is probably a major serotype in invasive disease also in Mexico. In the present report, the inability of the HVC to grow at 40°C was used as a marker to identify it in a collection of 57 GBS strains isolated from Mexico. Three serotype III invasive strains were identified as HVC. The growth of the other eleven invasive isolates and all the carrier isolates, regardless of serotype, was unaffected at 40°C. These results demonstrate that the HVC of GBS exists in Mexico, but it appears to be of low prevalence. Such a low prevalence could account for the low rate of invasive neonatal disease caused by GBS in Mexico.

Materials and Methods

Bacteria. Strains used in this study have been described in detail elsewhere [17]. Briefly, 57 GBS strains isolated in Mexico were examined, 43 from asymptomatic adult and infant carriers, and 14 from infants and women with invasive disease (sepsis, meningitis, pneumonia, endometritis, and an abscess). Serotype III GBS strains 110 (division I, HVC [15]) and D136c (division II, asymptomatic/avirulent [15]), described in detail elsewhere, were used as controls [11, 12, 15, 17, 27]. Strains were no more than two passages from the clinical situation.

Serotyping. Strains were serotyped by the capillary precipitin method with antigenic extracts prepared from whole cells by the Lancefield hot-HCl method [6, 17], and an enzymatic procedure using *N*-acetylmuramidase (mutanolysin) as previously described [6, 17]. Nineteen isolates were serotype III, and the remaining ones were of other serotypes including some nontypable strains (Table 1).

Growth media. Every strain was stocked in Todd-Hewitt broth (Difco) and stored at −70°C. Thawed cultures were routinely streaked onto 5% sheep blood agar plates (BBL Microbiology Systems, Cockeysville, MD) and incubated at 37°C overnight before each experiment. All growth studies were performed with a chemically defined medium (FMC) containing 65 mM sodium phosphate [14, 23]. This medium was modified by adding whole Todd-Hewitt broth (Difco) at different concentrations (10–1000 μg/ml) to study six strains growing slowly in FMC.

Table 1. Serotypes of 57 Mexican GBS strains as determined by the capillary precipitin test using antigens prepared by both the hot-acid extraction and an enzymatic procedure [17]

Serotype	No. of strains		
	Carrier isolates	Invasive isolates	Total
III	10	9	19
II	7	1	8
Ia	6	2	8
Ib	8	2	10
Ic	7		7
NT[a]	5		5
Total	43	14	57

[a] NT, nontypable.

Growth conditions and temperature shift experiments. Growth conditions and temperature shift experiments were basically as previously described [12]. Growth was monitored by measuring the A_{675} in a spectrophotometer (Junior model 35; The Perkin-Elmer Corp., Norwalk, CT). The optical density was converted to adjusted optical density units (AOD) and multiplied by 1000. Organisms were inoculated from fresh blood agar plates in 10 ml of FMC and grown aerobically at 37°C in a circulating water bath. Starting inoculum was adjusted to 30–40 AOD. Cultures were immediately chilled in an ice bath when they reached 400–500 AOD (mid-exponential phase). Then, two 10-ml tubes of freshly made FMC were inoculated with about 0.625 ml of the initial culture and incubated at 37° and 40°C. Starting inoculum was 20–30 AOD. Growth at both temperatures was monitored every 30 min for 3.5 h. Inability to grow at 40°C was defined as a less than twofold increase in cellular mass during this time period. Each strain was assayed at least twice.

Statistical analysis. The significance of differences in AOD between strains whose growth was and was not inhibited at 40°C was evaluated by using the nonparametric Mann-Whitney *U* test. Differences of growth at 37° and 40°C of strains classified and not classified as HVC were determined by the nonparametric Wilcoxon signed-rank test. $p < 0.05$ was considered statistically significant [10].

Results

Growth of all our strains isolated from asymptomatically colonized patients, regardless of the serotype, was unaffected by increasing the growth temperature to 40°C (Fig. 1). In contrast, the growth of strain 110, a serotype III isolated from an infant with meningitis [14] and representative of the HVC (Musser's division I [15]), was inhibited at 40°C. Strain D136c, also serotype III but known to be avirulent [14] and representative of the Musser's division II [15], had an unaffected growth when culture temperature increased. One representative run of some isolates was selected to show the growth pattern of

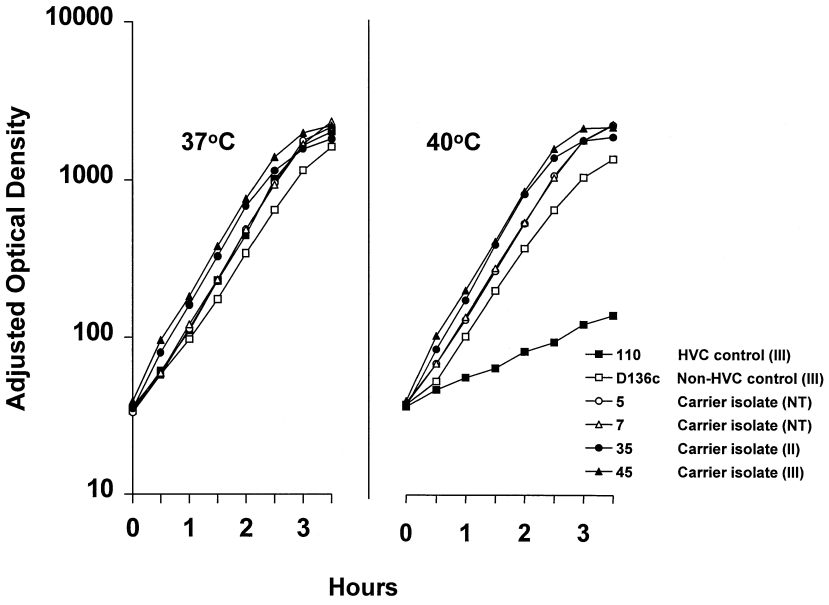

Fig. 1. Growth in FMC medium at 37° and 40°C of four Mexican GBS strains isolated from asymptomatic carriers. Strains 110 (HVC, infected neonate, division I [15]) and D136c (avirulent isolate, division II [15]) were used as controls. Number in parentheses is the serotype for each strain. Only one representative run of each strain is plotted.

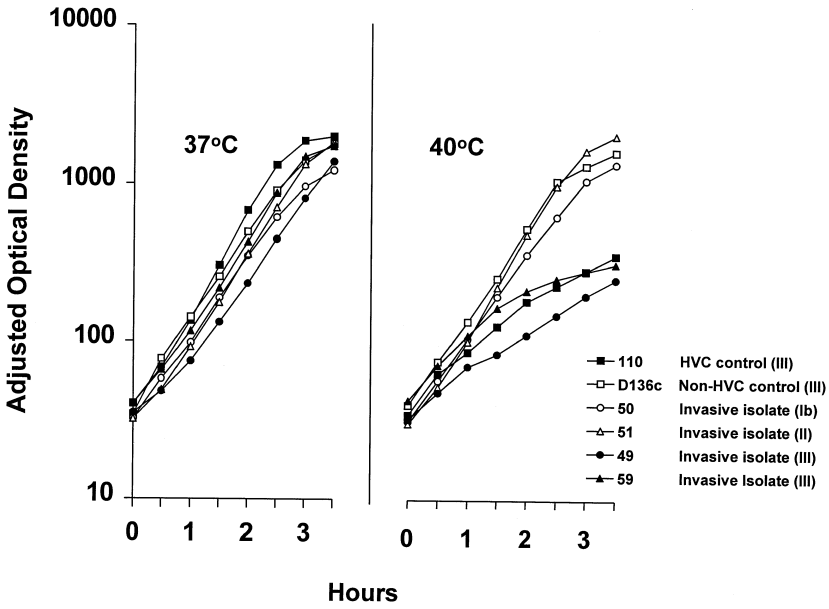

Fig. 2. Growth in FMC medium at 37° and 40°C of four Mexican GBS strains isolated from invasive disease cases. Number in parentheses is the serotype for each strain. Only one representative run of each strain is plotted.

asymptomatic carrier and invasive disease isolates in Figs. 1 and 2.

Invasive isolates showed two growth patterns. Three serotype III invasive isolates (strains 49, 59, and 62) whose growth was inhibited at 40°C, had growth curves similar to that of the HVC strain 110 (Fig. 2). The other 11 invasive strains, independent of serotype (Table 1), had growth curves unaffected by the increase in the growth temperature (Fig. 2). It was noteworthy that most (10/11) of our invasive isolates having growth curves unaffected by the temperature increase had at least one perinatal factor increasing the susceptibility to infection in the newborn, such as premature delivery (6/11), fetal hypoxia (6/11), maternal peripartum infection or fever (6/11), intra-amniotic infection (4/11), premature rupture of membranes (3/11), and difficult delivery (2/11). In contrast, just one of the three invasive isolates whose growth was inhibited at 40°C (strain 49) had premature delivery as a single perinatal high risk factor.

AOD of isolates whose growth was and was not inhibited at 40°C (HVC and non-HVC)—213 ± 111 and 1540 ± 375 (mean ± SD at 3.5 h)—were statistically different ($p = 0.000000$ Mann-Whitney U test) (Fig. 3). Although AOD of these isolates were statistically differ-

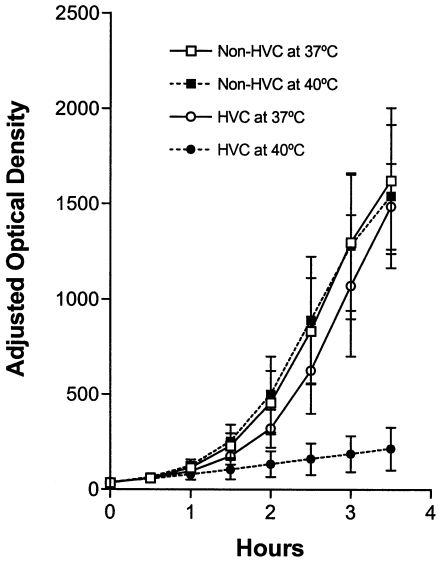

Fig. 3. Comparison of growth (mean ± SD of AOD) at 37° and 40°C in FMC of Mexican GBS isolates classified (n = 3) and not classified (n = 54) as being the HVC. A representative run of every strain was used to calculate means.

ent from 1 h of incubation (77 ± 27 vs. 123 ± 32, $p = 0.02$ Mann-Whitney U test), a more than twofold difference was found by 1.5 h (104 ± 51 vs. 252 ± 87). Furthermore, growth difference at 37° and 40°C of strains classified as being the HVC (1486 ± 225 and 213 ± 67 at 3.5 h of incubation) was also significant ($p = 0.004$ Wilcoxon signed-rank test). On the other hand, there was no difference comparing growth at 37° and 40°C of strains not classified as HVC (Fig. 3).

Discussion

Although GBS are a major cause of neonatal sepsis and meningitis, they colonize the rectum and vagina of pregnant women usually without clinical consequences for the newborns. Why some infants and mothers develop disease and others remain asymptomatic has not yet been answered. Although the immune status of colonized mothers has been suggested to play a role in providing protection to their infants [1], a number of studies have suggested that differences in virulence of GBS isolates may also contribute to neonatal infection [1, 5, 9, 14–16, 20]. Similar observations in virulence differences have been reported for *Escherichia coli* and *Haemophilus influenzae* [18, 25]. In addition, results of other studies have suggested that isolates of a single clone or a small number of clones could account for much of the morbidity and mortality caused by GBS [9, 15, 19].

Isolates representing the HVC of GBS appear to have several unique phenotypic characteristics, such as the elaboration of high levels of extracellular type-specific antigen [5], extracellular hyaluronidase [14], and cell-associated lipoteichoic acid [16]. These findings were followed by efforts to develop specific procedures for rapid identification of this HVC. Thus, several other unique characteristics of this clone were reported, such as its inability to grow at 40°C [12] and in a medium containing 200 mM phosphate [13]. Furthermore, upon examination of the molecular basis for the inability of this clone to grow at 40°C, a temperature-sensitive fructose-1,6-biphosphate aldolase was identified [11]. This aldolase lost 75% of its enzymatic activity after pre-incubation at 40°C compared with 37°C. This characteristic has been used as a highly specific and sensitive marker to identify the HVC of GBS [11, 24].

Further population genetics studies have attempted to identify a high virulence clone in a number of collections of GBS isolates from different countries [2, 9, 15]. Blumberg et al. [2] used restriction endonuclease analysis of chromosomal DNA and ribotyping to subtype 76 GBS isolates. They found four ribotypes among invasive serotype III isolates, suggesting genetic heterogeneity, and proposed that invasive serotype III isolates may not belong to a single clone. However, in a study of 118 GBS Danish isolates [9] and another of 276 French isolates [19], evaluation of the chromosomal genotypes by multi-locus enzyme electrophoresis found a phylogenetic dichotomy among serotype III isolates. In the cluster analysis, two primary phylogenetic divisions were found in both studies. Both divisions contained serotype III isolates, but one comprised virtually all invasive disease isolates, and the other exclusively isolates from healthy pregnant women. Furthermore, although serotype III isolates showed the greatest genetic diversity, most of the invasive serotype III isolates clustered in a limited number of electrophoretic types. These results supported the clonal structure of the GBS populations studied and the differences in virulence among clones. In addition, they demonstrated the existence of an unlimited number of ‘‘carrier clones’’ of serotype III and other serotypes, and a restricted number of clones of apparent worldwide distribution that cause invasive infections. Nevertheless, more recently Hauge et al. [8] used additional typing methods to characterize Helmig’s Danish strain collection, including six North American strains from Musser’s study [15]. Twenty-three genetic and phenotypic characteristics were evaluated, including the 11 metabolic loci Musser studied. They found six major phylogenetic divisions and a strong correlation between individual characters evaluated, indicating a clonal structure of the population. Moreover, like Musser and Helmig, Hauge found that strains expressing type III polysaccharide belonged to two distantly related evolutionary lineages of

clones. However, they found no evidence for differences in pathogenic potential among these divisions, but the number of strains evaluated was small compared with the number of variables they analyzed, and they did not control for high-risk perinatal factors. Thus, in spite of the aforementioned studies, the relationship between infection and the genetic structure of the GBS population remains unclear.

Although studies evaluating the role of GBS in perinatal disease in Mexico are limited, it has been shown recently that type III is a major serotype in invasive disease in Mexico as well [17]. In addition, in a nationwide GBS sero-epidemiologic survey, a sero-prevalence of 90% in 2669 serum samples tested demonstrated that the Mexican population has a high rate of exposure to GBS (in preparation). In light of these findings, examination for the HVC of GBS in a collection of strains isolated in Mexico was undertaken, with the inability of this clone to grow at 40°C used as a marker, as previously described [12].

The growth of 3 of 14 invasive isolates in the present study was reduced significantly when growth temperature was increased. Thus, these isolates were classified as members of the HVC. The other 11 invasive isolates, most of them serotype III, had growth curves unaffected at 40°C. In addition, none of our asymptomatic carrier isolates had growth curves affected by the increase in culture temperature. Furthermore, as in previous reports, all isolates identified as being the HVC were serotype III [12, 15]. These results demonstrate that the HVC, identified through its inability to grow at 40°C, exists in Mexico, but may be of low prevalence. This low prevalence, in light of the phenotypic characteristics of this clone indicating an elevated virulence, could be responsible in part for the low rate of neonatal invasive disease due to GBS in Mexico. However, a significant number of invasive disease cases were caused by strains that did not belong to the HVC. Since most cases of disease caused by such strains had additional high-risk perinatal factors, it is likely that conditions such as premature delivery, premature rupture of membranes, fetal hypoxia, and difficult delivery could have increased the invasive potential of strains otherwise of low virulence. This is possible by a number of mechanisms, including rupture of mucous membrane barriers against infection, bacterial inoculum increases, and reduction of the immune response of the host [1]. These factors are prevalent in developing countries where considerable limitations in prenatal care and delivery are common. In addition, they could also account for the high case-fatality rate previously reported in Mexico [22]. Studies evaluating the role of the HVC of GBS in perinatal pathology controlling for such high-risk perinatal factors are lacking.

On the other hand, the role of local environment and factors particular to the Mexican population in the low prevalence of this HVC, even in GBS invasive disease as found in this study (3 of 14 invasive isolates), is not known. In addition, it is not clear whether the prevalence of this HVC will increase eventually in Mexico, as appears to have occurred in other countries, and whether such an event would increase the GBS disease rate [1, 7].

This is the first effort to try to identify the role of the HVC of GBS in perinatal pathology in Mexico, and to our knowledge the first one in Latin America. Our results suggest that this HVC, identified by its inability to grow at 40°C, has a low prevalence in Mexico, and that an important group of GBS invasive cases in Mexico is caused by strains other than this HVC, most likely owing to additional high-risk perinatal factors.

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